Carbohydrate Research, 209 (1991) 33–50
Elsevier Science Publishers B.V., Amsterdam

Fast-atom-bombardment mass spectrometry of sulphated oligosaccharides from ovine lutropin

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(Received March 10th, 1990; accepted for publication, May 17th, 1990)

ABSTRACT

The positive- and negative-ion f.a.b.-mass spectra and the fragmentation of sulphated oligosaccharides derived from ovine lutropin are described. Negative-ion f.a.b.-m.s. of methylated derivatives offers a sensitive and rapid method for screening glycans for sulphation, for defining the location of sulphated residues, and for sequencing sulphated branches. Positive-ion f.a.b.-m.s. gives complementary data on non-sulphated branches in both complex and hybrid-type sulphated structures.

INTRODUCTION

Luteinizing hormone (LH), follicle-stimulating hormone (FSH), and thyroid-stimulating hormone (TSH), produced by the anterior pituitary gland, are heterodimeric glycoproteins, composed of a common α-subunit and a hormone-specific β-subunit[1]. Their exclusively Asn-linked hybrid- and complex-type oligosaccharide chains, which are essential for complete expression of biological activity, terminate with either sialylated Gal or unique GalNAc-4-SO_4, or both, in the peripheral branches[2].

The rigorous characterisation of the structures of the hybrid- and complex-type glycans present at each site of glycosylation in pituitary glycoproteins is difficult and is further complicated by the intractability of sulphate residues to many structural techniques. F.a.b.-m.s. is potentially a powerful tool for the analysis of these molecules. In a variety of applications to non-sulphated glycoproteins, we[3–7] and others[8,9] have shown that the technique can define all of the non-reducing structures present in heterogeneous mixtures of glycans, provided that it is applied to suitable derivatives, most notably the methylated derivative. Positive-ion f.a.b.-mass spectra of methylated N-glycans contain characteristic arrays of fragment ions which are derived from cleavage at each amino sugar linkage with the charge being retained on the non-reducing fragment[10]. These ions constitute a highly reproducible and sensitive fingerprint of the non-reducing moieties. Clearly, it would be of value if sulphated oligosaccharides were amenable to this type of analysis.

Polysulphated oligosaccharides derived from glycosaminoglycans are stable un-

0008-6215/91/$03.50

der mild conditions of methylation[11]. We now report that these procedures are applicable to the sulphated bi-antennary N-glycans present in pituitary glycoproteins and that, at the μg level, the methylated products yield negative-ion f.a.b.-mass spectra of high quality. These spectra contain characteristic fingerprints of fragment ions which are obtained reproducibly from each molecule that contains a sulphated branch irrespective of the composition of the second branch or the nature of the reducing end. We also report on the fragmentation of these molecules in the positive-ion mode.

EXPERIMENTAL

Materials. — SepPak cartridges were purchased from Waters Ltd.

Preparation of oligosaccharides. — Sulphated oligosaccharides were prepared from ovine lutropin provided by the National Hormone and Pituitary Program of the National Institute of Arthritis, Diabetes, and Digestive and Kidney Disease (U.S.A.). The sugar chains were released by hydrazinolysis[12], then re-*N*-acetylated and reduced with $NaBH_4$. The resulting oligosaccharide-alditols were fractionated by anion-exchange and ion-suppression amine-adsorption h.p.l.c. as described[13].

Methylation. — A modified Hakomori procedure[14,15] was used. Solutions of samples ($\sim$10 μg) each in dried, distilled methyl sulphoxide (100 μL), to which was added a solution (100 μL) of methylsulphinylmethanide prepared by mixing sodium hydride (0.1 g) with methyl sulphoxide (400 μL), were incubated at 100° for 10–15 min, then centrifuged for 10 min at 2000*g*. Each sample was stored at room temperature for 5 min, methyl iodide (100 μL) was added, and the reaction was continued for 20 min. The sequential additions of the base and methyl iodide (400 μL of each) were repeated. The reaction was stopped after 20 min by the addition of distilled water (0.5 mL), and excess of methyl iodide was removed in a stream of nitrogen. A solution of each vacuum-dried residue in 1:1 methanol–water (200 μL) was applied to a conditioned SepPak cartridge[15] and eluted with aqueous 30% acetonitrile.

F.a.b.-m.s. — A VG Analytical ZAB-HF mass spectrometer, fitted with an M-Scan f.a.b. gun operating at 10 kV with a beam strength of 15 μA, was operated at an accelerating voltage of 8 kV. Each sample was dissolved in methanol prior to analysis. The matrix was glycerol–thioglycerol (1:1). Data were recorded on oscillographic paper and manually counted. The spectra as recorded were not suitable for direct photographic reproduction and were copied by tracing manually.

RESULTS

The oligosaccharides studied were **1**–**4**. The structures of **1**–**3** have been determined by 1D and 2D ^{1}H-n.m.r. spectroscopy[13]. The oligosaccharides were released from ovine lutropin by hydrazinolysis, then re-*N*-acetylated, reduced, and purified by h.p.l.c. prior to methylation and f.a.b.-m.s..

Negative-ion f.a.b.-m.s. of methylated **1**. — The spectrum, reproduced in Fig. 1, contains an intense $[M - H]^-$ ion at *m/z* 2127, which is at the nominal mass calculated

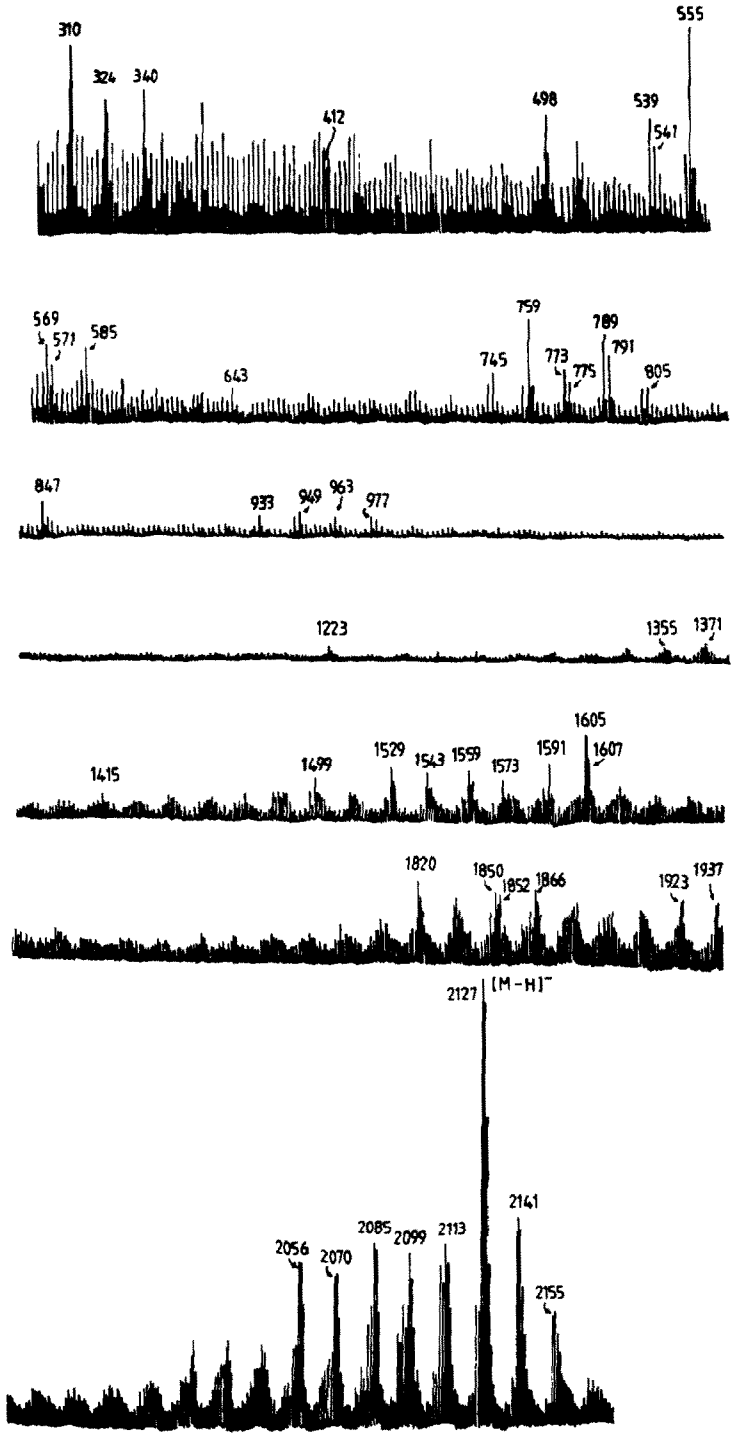
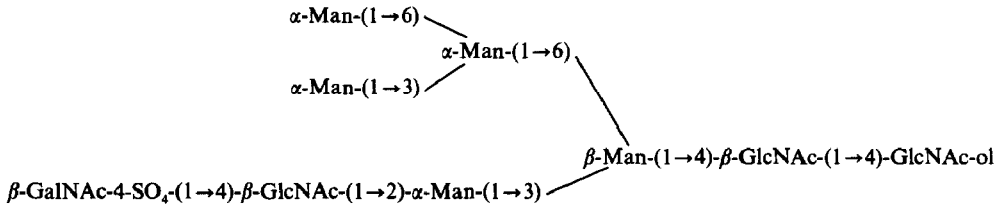


Fig. 1. Negative-ion f.a.b.-mass spectrum of methylated **1**.



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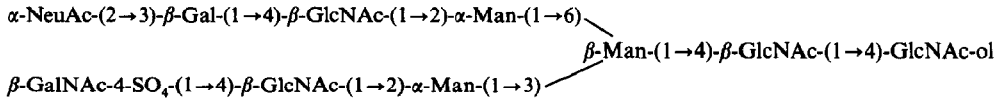

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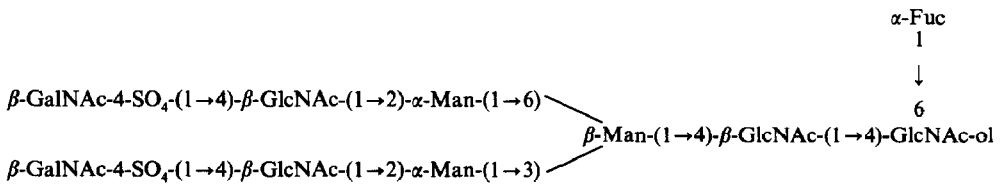

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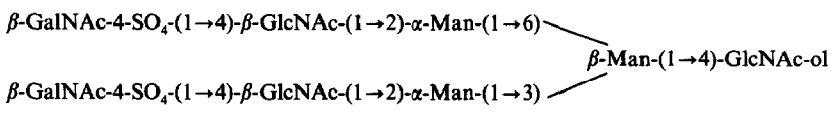

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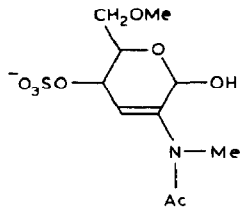
for fully methylated **1**. The clusters of signals at intervals of 14 m.u. lower than that of the major molecular ion are derived from undermethylated components. This incomplete methylation involved mainly the reducing-end GlcNAc and did not affect fragmentation from the non-reducing end of the molecule. The low-mass region of the spectrum contains clusters of fragment ions which are separated from each other by increments of sugar residues. The cluster of lowest mass, which is derived from the terminal GalNAc-4-SO_4, contains major signals at *m/z* 310, 324, and 340, and a minor satellite at *m/z* 412. Possible structures for these fragment ions are **5**–**7** and **9**.

Structures **5**–**7** can be rationalised as arising from cleavage of the linkage between GalNAc-4-SO_4 and the adjacent GlcNAc together with hydrogen transfer and/or elimination of functional groups. Ring cleavage (**8**) of the GlcNAc residue yields structure **9** in which C-4,5,6 are retained in the fragment ion.



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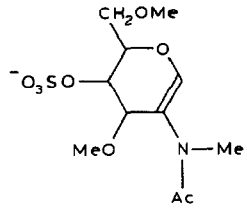

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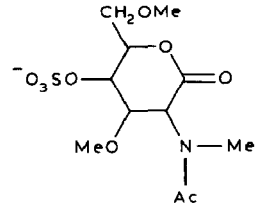

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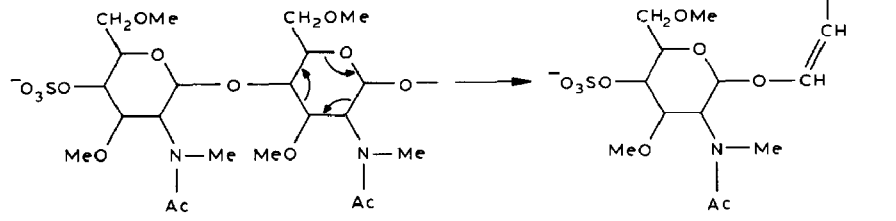

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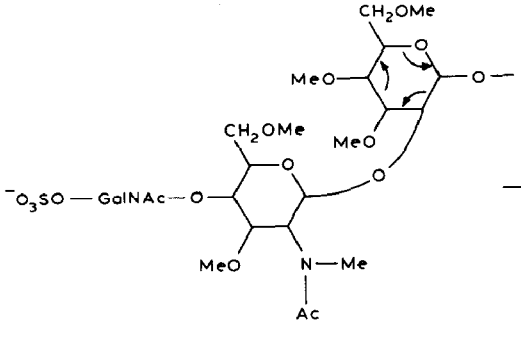
Fragmentation at the second linkage, between GlcNAc and Man, yields the ion cluster in the region *m/z* 498–643. Major signals at *m/z* 555, 569, and 585 are 245 m.u. (the HexNAc increment) higher than those at *m/z* 310, 324, and 340, respectively. The satellite signal at *m/z* 643 is consistent with a 2-linkage of the GlcNAc to Man, which precludes formation of the analogue of **9**. A plausible structure for the *m/z* 643 ion is **11**, possibly formed via a ring cleavage reaction (**10**). Additional prominent signals in the region near *m/z* 500 occur at *m/z* 498, 539, and 571, and are assigned the structures **12–14**, respectively.



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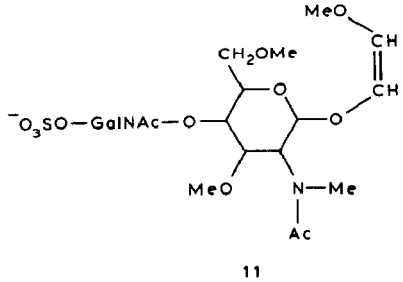

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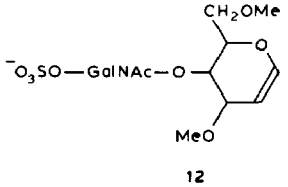

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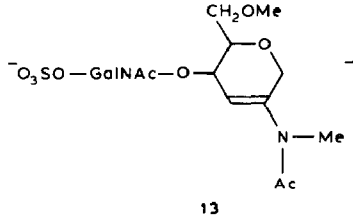

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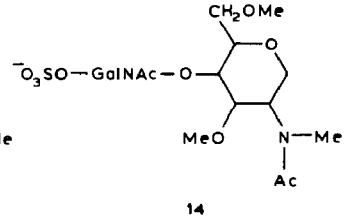

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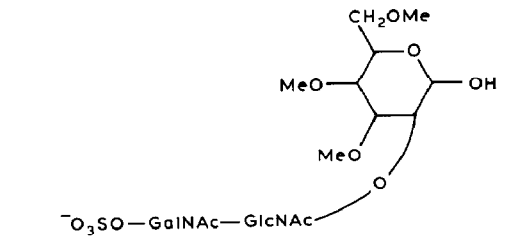
The next prominent ion cluster extends from *m/z* 745 to 847. Most of the signals in this cluster are 204 m.u. (the Hex increment) higher than major signals in the GlcNAc cluster, and are attributed to fragmentation between the Man residue in the sulphated branch and the disubstituted Man of the core. An additional signal at *m/z* 791 is assigned the structure **15**. Ring cleavage (**16**) of the 3,6-linked Man yields the satellite signal at *m/z* 847 (**17**).



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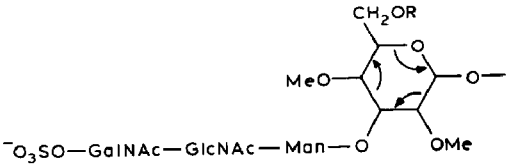

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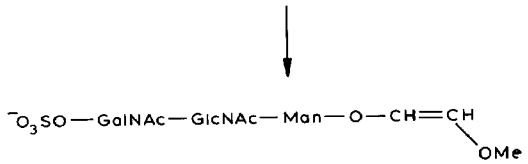
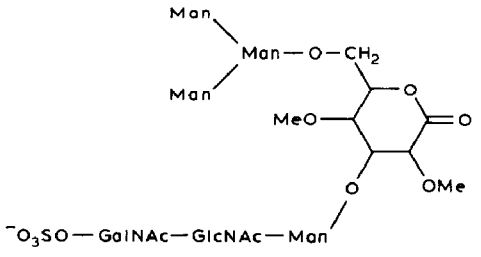


17

At *m/z* >850, there is a marked decrease in the abundance of fragment ions up to the region *m/z* 1500–1600. The cluster of weak signals near *m/z* 950 is similar in appearance to that near *m/z* 800 and is separated therefrom by the mass (190 m.u.) of an "undermethylated" hexose. This finding is suggestive of a branched molecule that produces fragment ions by multiple cleavage events. The complex set of ions near *m/z* 1600 is the result of cleavage between the 3,6-linked Man and the first GlcNAc of the



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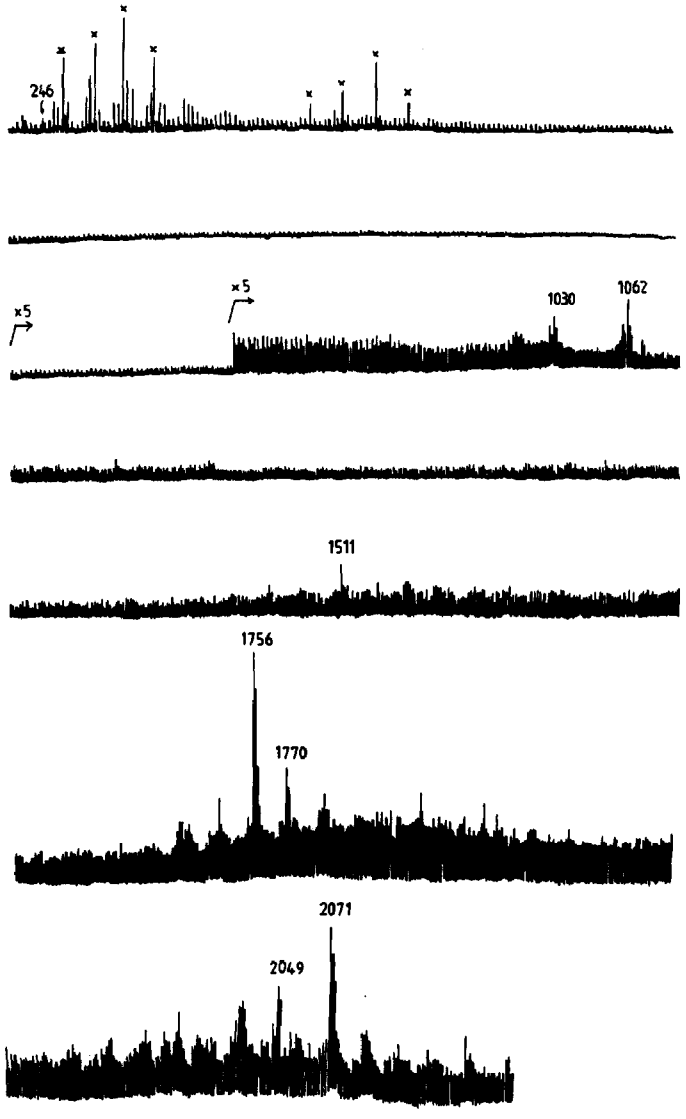
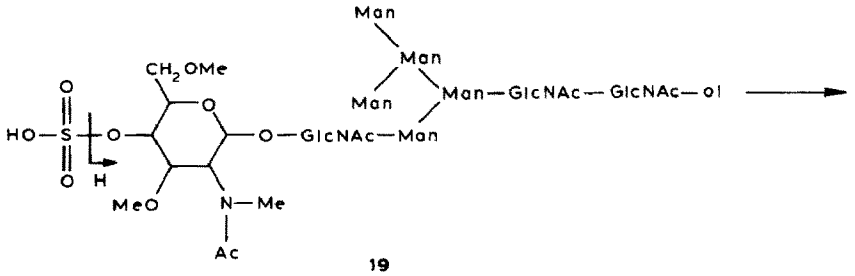


Fig. 2. Positive-ion f.a.b.-mass spectrum of methylated **1**.

core. For example, the most prominent ion in the cluster, *m/z* 1605, is separated from that at *m/z* 789 by 816 m.u. (a Hex_4 increment), and therefore the ion at *m/z* 1605 is assigned the structure **18**. Similarly, the ions at *m/z* 1591 and 1607 are 816 m.u. higher than those at *m/z* 775 and 791, respectively. Other ions in the cluster are 32 m.u. less than the predicted masses for a Hex_4 increment from the ion cluster near *m/z* 700. These ions arise by elimination of methanol, most likely from position 2 of Man, concomitant with cleavage of the linkage. The ion cluster near *m/z* 1850, which is formed by fragmentation between the two GlcNAc residues of the core, can be rationalised as for all of the lower-mass-clusters.

Positive-ion f.a.b.-m.s. of methylated **1**. — In the spectrum shown in Fig. 2, none of the ions carries a sulphate moiety. In the region for molecular ions, the two major signals at *m/z* 2049 and 2071 correspond to $[M + H]^+$ and $[M + Na]^+$, respectively, for the hybrid molecule **20** formed by loss of sulphite in a *β*-cleavage reaction[10] (**19**).



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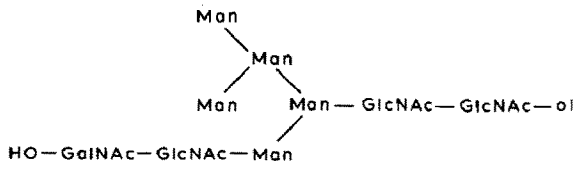

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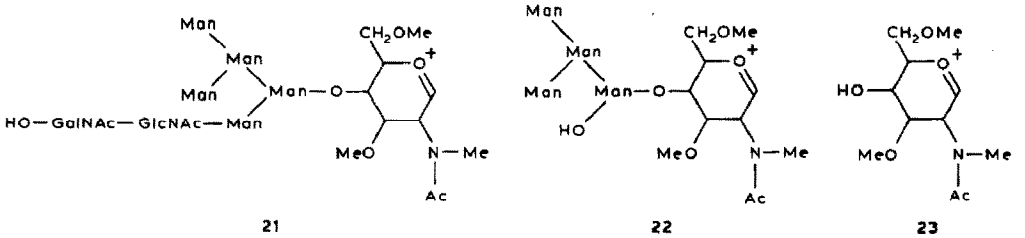
The fragmentation pattern is similar to those described for methylated N-glycans[10]. The intense signal at *m/z* 1756 is consistent with A-type fragmentation in the chitobiose core, concomitant with loss of sulphite by *β*-cleavage to give structure **21**. A similar double-cleavage reaction, that involves *β*-cleavage at the core mannose, yields structure **22** (*m/z* 1062) which can lose methanol to produce a signal at *m/z* 1030. The terminal GalNAc-4-SO_4 gives a weak signal at *m/z* 246 consistent with structure **23**.



21 **22** **23**

Negative-ion f.a.b.-m.s. of methylated **2**. — The spectrum (Fig. 3) contains a signal for a major molecular ion at *m/z* 2529 which is at the mass expected for methylated **2**. Fragmentation occurs exclusively in the sulphated branch to give clusters of fragment ions that contain the sulphate moiety. These clusters are identical to those present at *m/z* < 1000 in the spectrum of methylated **1**.

Positive-ion f.a.b.-m.s. of methylated **2**. — In the spectrum shown in Fig. 4, the minor signal at *m/z* 2473 corresponds to $[M - SO_3 + Na]^+$ and is accompanied by a

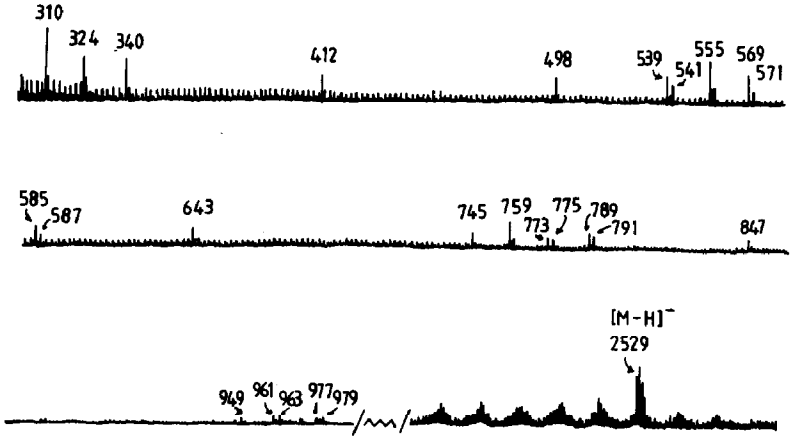


Fig. 3. Negative-ion f.a.b.-mass spectrum of methylated **2**.

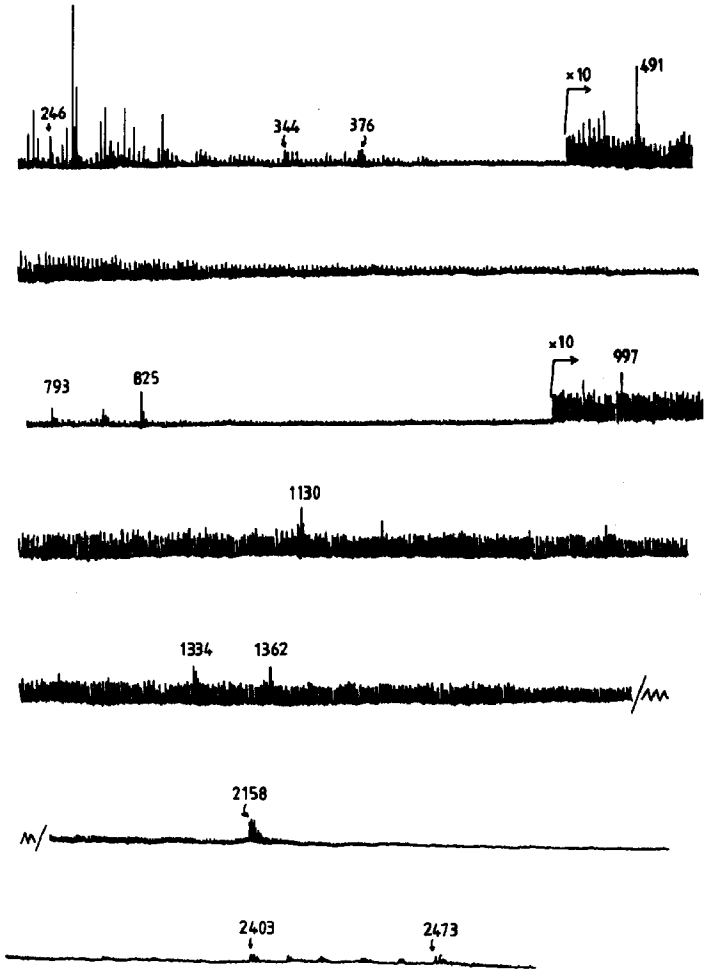
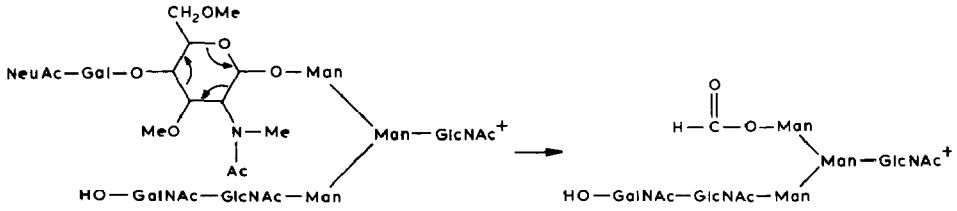


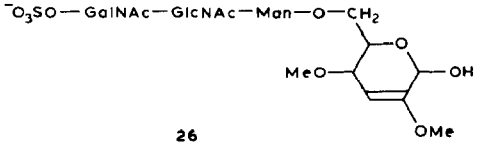
Fig. 4. Positive-ion f.a.b.-mass spectrum of methylated **2**.

series of signals at lower mass due to several degrees of undermethylation. The major fragment ions in the remainder of the spectrum are derived from A-type cleavage at each amino sugar residue, some of which are accompanied by loss of part or all of the sulphated branch by β-cleavage. The sialylated branch is characterised by ions at m/z 344 (376 − MeOH), 376 (NeuAc$^+$), 793 (825 − MeOH), 825 (NeuAcHexHexNAc$^+$), and 997 (NeuAcHexHexNAcHex$^+$ − MeOH). The sulphated branch yields the ions m/z 246 (HO–HexNAc$^+$) and 491 (HO–HexNAc–HexNAc$^+$). Cleavage of the chitobiose core together with loss of sulphite gives the ion m/z 2158 which is analogous to that (**21**) with m/z 1756 in Fig. 2. Additional losses of NeuAc–Gal–GlcNAc and NeuAc–Gal–GlcNAc–Man from the ion m/z 2158 afford the ions m/z 1334 and 1130, respectively. These signals are each accompanied by signals 28 m.u. higher, at m/z 1362 and 1158, respectively, which are derived from the ring-cleavage reaction[10] (**24**→**25**) that results in a formyl group being attached to the glycosidic oxygen involved in the fragmentation.



24 **25** m/z 1362

Negative-ion f.a.b.-m.s. of methylated **3**. — The spectrum in Fig. 5 contains a weak $[M + Na - 2H]^-$ signal at m/z 2471 and a strong signal due to loss of sodium sulphite at m/z 2369. Each of these signals is accompanied by a prominent signal 70 m.u. lower, which corresponds to five degrees of undermethylation. The undermethylation appears to involve the reducing end of the molecule because the fragment ions derived from the branches do not show significant undermethylation. The ion $[M - 2H]^{2-}$ gives the signals near m/z 1200. The low-mass region of the spectrum is dominated by clusters of signals that are almost identical with those present in Figs. 1 and 3. However, there are some notable differences. First, instead of the weak cluster of multiple signals near m/z 960 present in Figs. 1 and 3, there is one clear signal at m/z 963 which is probably derived from elimination of the 3-linked branch together with β-cleavage between the 3,6-linked Man and the GlcNAc of the core to give structure **26**. Second, the pattern of signals near m/z 850 in Fig. 5 is very different from those in Figs. 1 and 3. Instead of a single satellite



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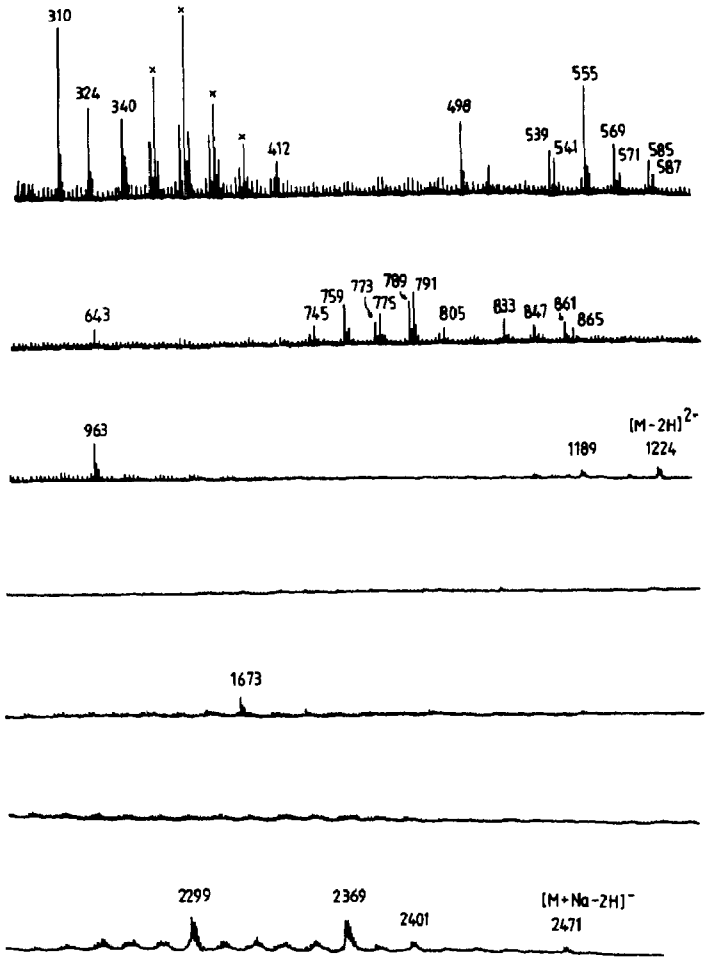
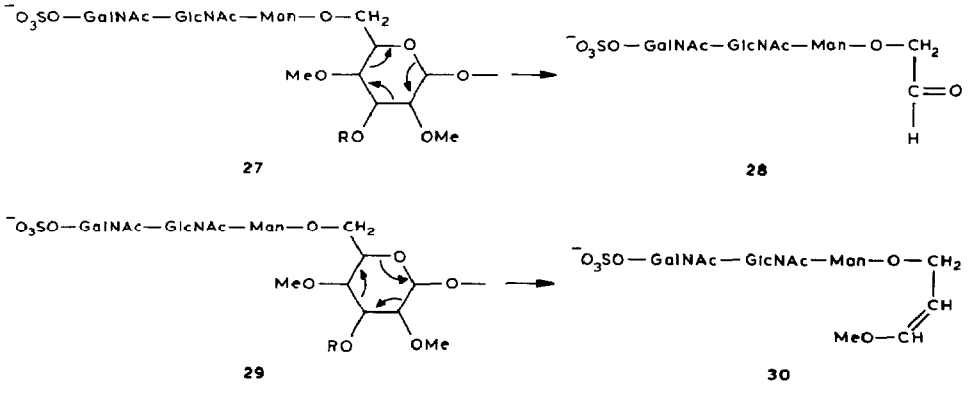


Fig. 5. Negative-ion f.a.b.-mass spectrum of methylated **3**.

signal at *m/z* 847, there is a triplet at *m/z* 833, 847, and 861. The additional ions of *m/z* 833 and 861 are assigned structures **28** and **30**, respectively, formed from **27** and **29**, respectively.



Positive-ion f.a.b.-m.s. of methylated **3**. — The spectrum in Fig. 6 contains a pair of signals at *m/z* 2243 (undermethylated 2313) and 2313 that are assigned to $[M + Na]^+$ adducts of methylated **3** from which both sulphite moieties have been removed by β-cleavages. Fragment ions identical to those observed in the spectrum of methylated **2** are present at *m/z* 246, 491, 1130, 1158, 1334, and 1362. All of these ions are derived from a sulphated branch or from one sulphated branch plus part of the core. High-mass fragment ions, which are not common to methylated **2** and **3**, carry both branches. These ions occur at *m/z* 1579 and 1824, and are assigned structures **31** and **32**, respectively. In the low-mass region of the spectrum, the signals at *m/z* 571 (**33**), 593

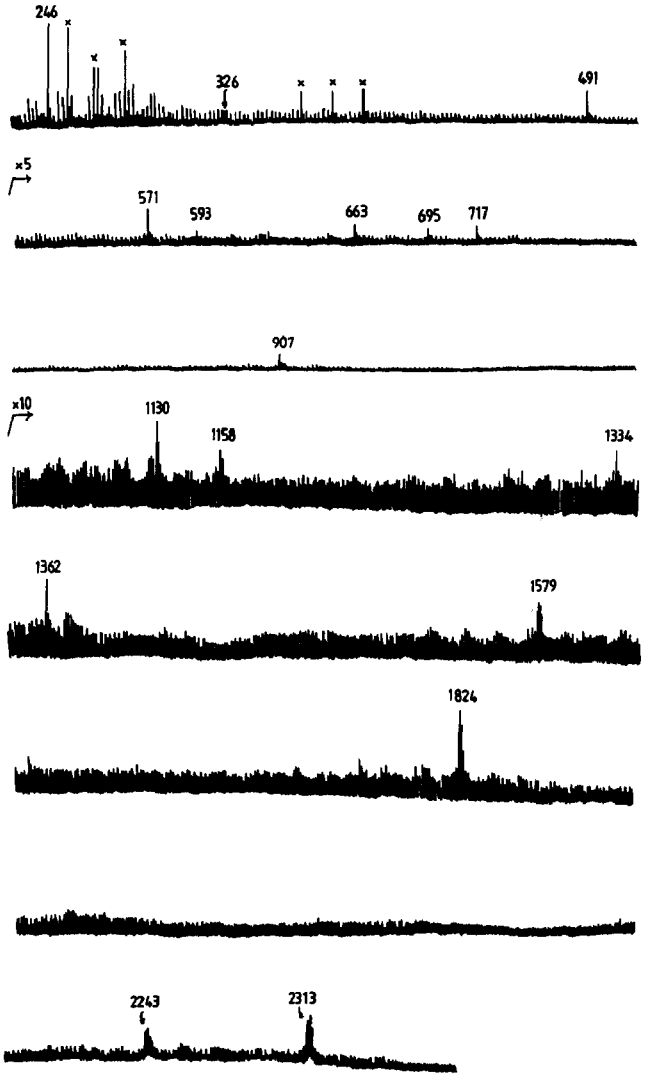


Fig. 6. Positive-ion f.a.b.-mass spectrum of methylated **3**.

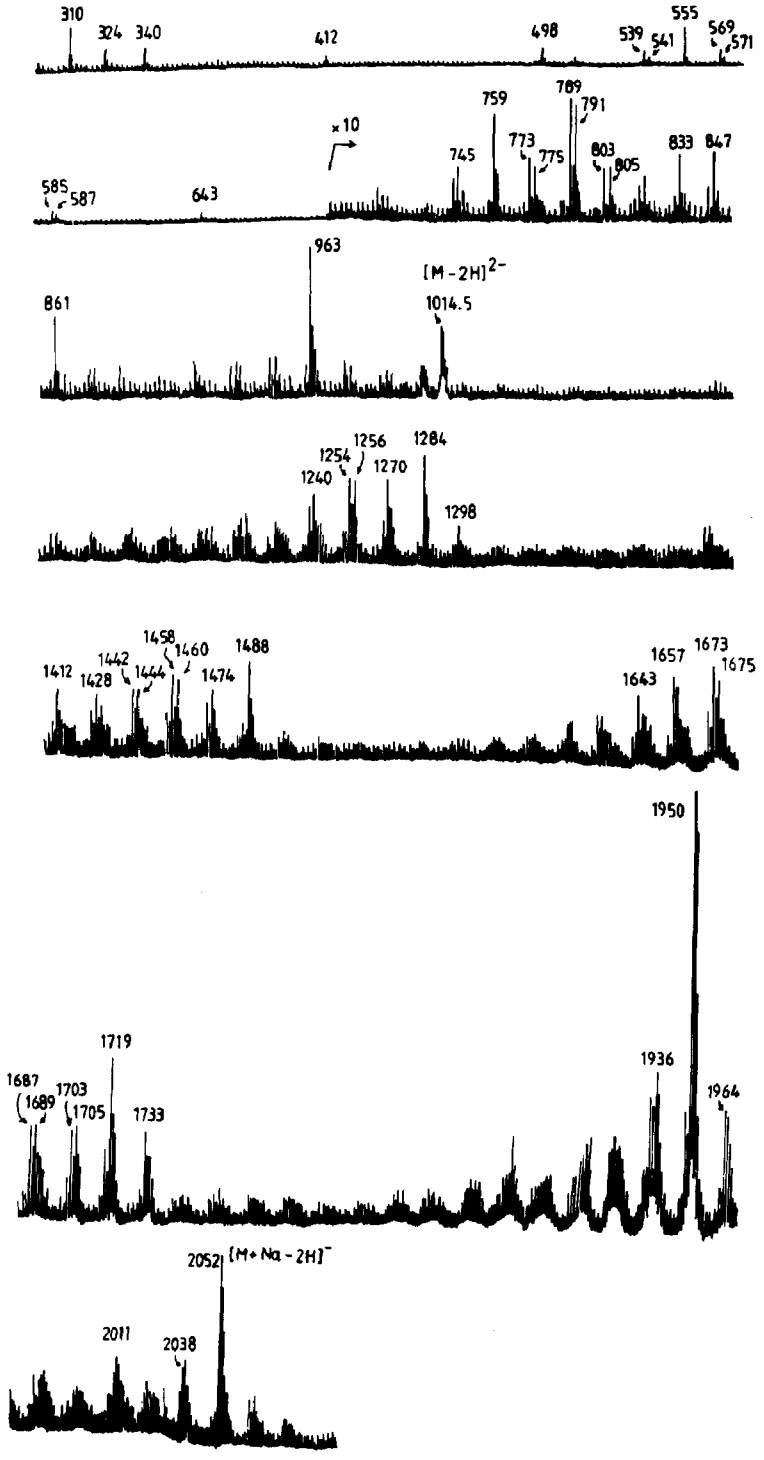
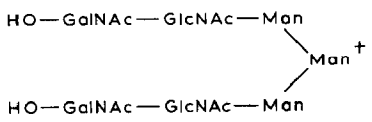


Fig. 7. Negative-ion f.a.b.-mass spectrum of methylated **4**.

(Na^+ salt of 571), 663 (695 − MeOH), 695 (**34**), and 717 (Na^+ salt of 695) are attributed to fragment ions each of which retains one sulphate group.



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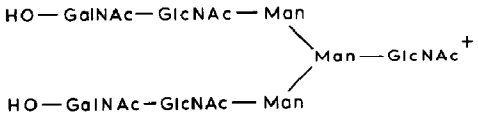

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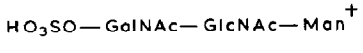
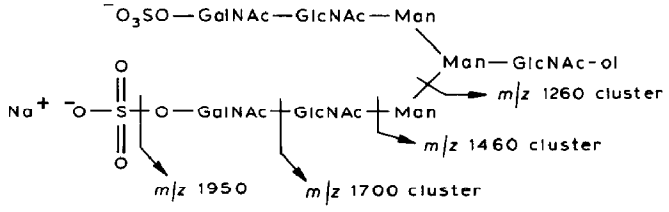


34

Negative-ion f.a.b.-m.s. of methylated **4**. — In the spectrum in Fig. 7, singly and doubly charged molecular ions at *m/z* 2052 and 1014.5, respectively, correspond to methylated **4**, which lacks the reducing-end GlcNAc of the N-glycan chitobiose core. Truncation most likely occurred during the hydrazinolysis. Similar truncation has been observed in the products of hydrazinolysis of non-sulphated glycoproteins[16]. The intense signal at *m/z* 1950 is formed by loss of sodium sulphite from the ion *m/z* 2052. Fragment ions with $m/z < 1000$ are identical to those afforded by methylated **3**. At $m/z > 1000$, there are three prominent clusters near *m/z* 1260, 1460, and 1700, which are separated by increments for sugar residues, and comprise (**35**) one sulphated branch, together with the core and parts of the second branch. The complexity of each cluster is similar to that exhibited by clusters at $m/z < 1000$, and the pathways of fragmentation that lead to their formation are likely to be the same as those outlined above for methylated **1**.



35

Positive-ion f.a.b.-m.s. of methylated **4**. — The spectrum in the positive ion-mode was weak and reflected the difficulty in producing positive ions from a disulphated molecule and the absence of the complete chitobiose core where cleavage occurs preferentially in methylated samples. Fig. 8 shows the region of the spectrum that contains the major fragment ions. At low mass (data not shown), weak signals were present at *m/z* 246 (HO–GalNAc$^+$) and 591 (HO–GalNAc–GlcNAc$^+$). Signals in Fig. 8 are assigned as follows: *m/z* 1894 to $[M + Na]^+$ of methylated **4** that has free hydroxyl

Fig. 8. Positive-ion f.a.b.-mass spectrum of methylated **4**.

groups at the sulphate attachment sites, 1858 to undermethylated 1894, 1649 and 1404 to loss of GalNAc and GalNAcGlcNAc, respectively, from *m/z* 1894 by *β*-cleavage, 1677 and 1432 to a formyl increment on 1649 and 1404, respectively, as noted above for methylated **2**.

DISCUSSION

A characteristic feature of the negative-ion f.a.b.-mass spectra of methylated **1–4** is the presence of a reproducible set of clusters of fragment ions in the low-mass region, which are derived from the sulphated branch(es). The diagnostic fingerprint of a sulphated-GalNAc–GlcNAc–Man branch is illustrated in Fig. 9. The presence of clusters, rather than one dominant fragment ion, for cleavage at each linkage, indicates that several possible modes of linkage and ring cleavage (see, for example, structures

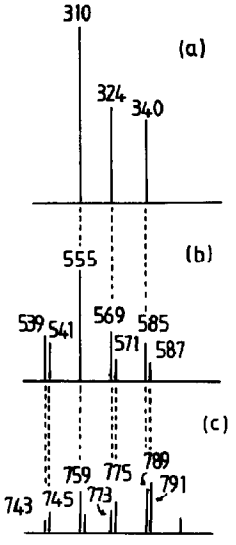


Fig. 9. Relationship between the diagnostic clusters of negative fragment ions which are formed by branches terminated by GalNAc-4-SO_4; (*a*) the cluster for GalNAc-4-SO_4, (*b*) the cluster for GalNAc-4-SO_4–GlcNAc, (*c*) the cluster for GalNAc-4-SO_4–GlcNAc–Man. The dotted lines connect signals separated by the mass of a sugar residue.

5–17) require similar internal energies. However, it is notable that linkage cleavage at the sulphated residue leads to a more limited set of fragment ions than linkage cleavage elsewhere. In contrast to the positive-ion f.a.b.-mass spectral behaviour of methylated glycans, cleavages at HexNAc and Hex appear to be favoured equally and result in similar sets of ions.

Our data suggest that the nature of the reducing end of the oligosaccharide has little influence on fragmentation of the antennae. This effect is high-lighted by methylated **3** and **4**, which produced identical sets of fragment ions at $m/z < 1000$. An additional feature of the spectra is the reproducibility of the fingerprint from the sulphated branch irrespective of the structure of the other non-reducing moieties. For example, the methylated hybrid structure **1** gave low-mass data identical to those afforded by the methylated sialylated structure **2**. These observations have important implications for the viability of negative-ion f.a.b.-m.s. as a general tool for screening glycoproteins or glycopeptides for the presence of sulphate and for the sequences of branches to which it is attached.

Each major set of fragment ions in the low-mass region of the negative-ion f.a.b.-mass spectra is accompanied by one or more satellite signals on the high-mass side of the cluster. These satellites are formed by ring-fragmentation of the glycosyl residue on the reducing side of the bond that cleaves to give the contiguous fragment-ion cluster. Linkage information is afforded by the masses of these satellite ions. For example, the ion m/z 412 (structure **9**) can be derived only from a 4- or a 6-linked residue. The latter could give **9** by the mechanism **29**→**30**. However, the data from methylated **3** and **4**, as exemplified by the ions m/z 833 and 861 (which are not given by methylated **1** and **2**, which do not carry a sulphated branch attached to the 6-position of Man), indicate that there are two equally favoured ring cleavages of 6-linked residues, which result in a pair of signals separated by 28 m.u. (*cf.* **27**→**28** and **29**→**30**). Thus, the satellite signals for the GalNAc-4-SO_4–GlcNAc–Man fragment ion are indicative of the position of attachment to the core Man: a single prominent signal at m/z 847 indicates attachment at position 3, whereas a 6-linked branch would give the ions m/z 833 and 861. Corroborative information on the linkage is provided by signals near m/z 960 which are due to ions that comprise one sulphated branch and the branched Man of the core. A weak, complex pattern of signals is afforded by methylated **1** and **2**. In contrast, methylated **3** and **4** give one major signal at m/z 963 which is rationalised as elimination of the substituent from position 3 of the Man (see structure **26**). Analogous cleavages of methylated **1** and **2**, although possible, would not yield a negatively charged species that contained the branching Man but would lead to structure **15**. It is concluded that sulphation on the 6-linked branch is characterised by satellite signals at m/z 833 and 861, and by a dominant signal at m/z 963, whereas sulphation of the 3-linked branch results in a weak, complex cluster near m/z 960, and a satellite signal at m/z 847.

Each of the sulphated glycans gave high quality positive-ion data. The positive-ion f.a.b.-mass spectra are characterised by "molecular ions" which carry free hydroxyl groups at the positions where the sulphate was attached. There is no evidence for loss of sulphate during methylation. Desulphated molecules would be expected to give abun-

dant $[M + H]^+$ ions at masses corresponding to fully methylated molecules, and no such signals are observed. It appears that loss of sodium sulphite occurs in the matrix during the f.a.b. process and subsequent fragmentation occurs via pathways that are common to all permethylated glycans. For example, the positive-ion f.a.b.-mass spectrum of methylated **2** contains a characteristic signal at m/z 825 which is diagnostic of a sialylated complex glycan[10]. The high-mass region of the positive-ion spectra of each compound that contained a complete core region is dominated by the A-type chitobiose cleavage ion[10].

The high quality of the f.a.b.-mass spectra acquired in this study, using sample loadings of 5–10 μg, attests to the potential power of the method for providing information on the structure of sulphated glycoproteins derived from a variety of biological sources. The widespread occurrence of sulphated glycoproteins is well documented[17–20], but, with the exception of the pituitary glycohormones[2], little is known of their detailed structure. Negative-ion f.a.b.-m.s. of methylated derivatives offers a sensitive and rapid method for screening for sulphation and defining the sequences of sulphated branches, including identification of the residues that carry sulphate groups. Positive-ion f.a.b.-m.s. of the same derivatives gives abundant fragment ions from non-sulphated branches, thereby providing complementary data. Together, the two modes of ionisation yield "maps" of fragment ions which define all non-reducing structures present in mixtures of sulphated and non-sulphated complex and hybrid oligosaccharides.

ACKNOWLEDGMENTS

Financial support from the Wellcome Trust and the Medical Research Council (to A.D. and H.R.M.) and from the Medical Research Council of New Zealand and Lottery Medical Research (to A.G.C.R.) is acknowledged, as is the U.G.C. for the award of a Postdoctoral Fellowship (to G.W.). The National Hormone and Pituitary Program of the National Institute of Arthritis, Diabetes, and Digestive and Kidney Disease (U.S.A.) is thanked for the generous gift of ovine lutropin.

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